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A protein specific to mitochondria from S-type male-sterile cytoplasm of maize is encoded by an episomal DNA

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Mitochondria of S-type cytoplasmic male sterile maize contain two linear double-stranded DNA molecules, S1 and S2. Two open reading frames (ORF1 and ORF2) are present in S2 DNA. Fragments from ORF1 were inserted into plasmids to achieve expression in *Escherichia coli*. Cells transformed with recombinant plasmids produced mRNA which hybridized with ORF1 and corresponding polypeptides were synthesized by *in vivo* and *in vitro* systems. Antiserum against a *lacZ*/S2 fusion protein precipitated the anticipated polypeptides from transformed *E. coli* cells and was therefore used to detect homologous peptide sequences in protein preparations of mitochondria from different maize cytoplasms. The antiserum detected a protein of 125 000 M_r present in mitochondria from male sterile B73S but absent from the fertile B73N cytoplasm.

Key words: maize/S-type/mitochondrial DNA/S2 fusion protein

Introduction

The mitochondrion of S-type cytoplasmic male sterile (cms-S) maize contains two linear double-stranded DNA molecules, S1 and S2, which are 6397 bp and 5453 bp in length respectively (Pring *et al.*, 1977). S1 and S2 have identical terminal inverted repeats of 208 bp and contain a further 1254 bp of homology adjacent to one of the terminal repeats (Levings and Sederoff, 1983; Paillard *et al.*, 1985). This region of homology contains an open reading frame, ORF2 (1017 bp) In addition S2 contains ORF1 (3513 bp) and S1 contains ORF3 (2787 bp) and ORF4 (768 bp) as shown in Figure 1.

Several lines of evidence have led to proposals that S1 and S2 may be mobile genetic elements and that they may also be responsible for the cms-S phenotype. These include the presence of terminal inverted repeats which are a feature of transposable elements of bacteria (Shapiro, 1983) and of eukaryotes, e.g. P and *hobo* elements of *Drosophila* (Finnegan and Fawcett, 1986), and the occurrence of sequences homologous to S1 and S2 in the main mitochondrial genome of both male sterile and male fertile maize lines (Thompson *et al.*, 1980; Lonsdale *et al.*, 1981, 1983), and sequences homologous to S1 in the nuclear genome (Kemble *et al.*, 1983). Transcripts from ORF1 and ORF2 are detected in mitochondria of S-type maize (Schardl *et al.*, 1985; J.C.Manson, unpublished observations), which also synthesize *in vitro* a number of high molecular weight polypeptides that are not detected in N mitochondria (Forde and Leaver, 1979). Whether S1 and S2 are directly responsible for the cms-S phenotype is not known, although the acquisition of this phenotype is accompanied by rearrangements of S1 and S2 homologous sequences in the mitochondrion (Schardl *et al.*, 1985).

It has been suggested that the mode of replication of S1 and S2 may be different from that of the mitochondrial genome. S1 and S2 are found in an approximately 5-fold copy number excess over sequences in the main mitochondrial genome (Laughnan *et al.*, 1981). They have protein attached to the 5′ ends of the DNA (Kemble and Thompson, 1982) and in this respect resemble a number of animal viruses, e.g. adenovirus (Rekosh *et al.*, 1977) and hepatitis B virus (Gerlich and Robinson, 1980) and bacteriophages, e.g. *Bacillus subtilis* ϕ29 (Mellado *et al.*, 1983) in which this protein may participate in the replication process. The mode of replication and function of S1 and S2 are, however, unknown. To learn more about these aspects of their biology we have expressed part of the open reading frame, ORF1 in *E. coli* and produced antibodies that have been used to identify cms-S specific proteins.

Results

Nucleotide sequence analysis of S2

Mitochondrial DNA was isolated from 5-day-old coleoptiles of maize line B73S and S2 DNA was purified by agarose gel electrophoresis and electroelution. Restriction endonuclease fragments of S2 DNA were cloned into M13 bacteriophage vectors mp8 and mp9 (Messing and Vieira, 1982) and the nucleotide sequence of S2 was obtained by the dideoxy chain termination procedure (Sanger *et al.*, 1977). This confirmed the sequence of Levings and Sederoff (1983) and analysis of the sequence using computer programs developed by Staden (1982) identified two open reading frames (Figure 1).

ORF1 (defined as the distance between termination codons) extends from base 180 to base 3692 with the first methionine starting at base 216 and it therefore has the potential to code for a protein of ~127 000 mol. wt. Restriction fragments from this open reading frame were inserted into plasmid vectors designed to give high levels of expression in *E. coli*.

Construction of recombinant plasmids

Two types of construct were used, one designed to give an S2 protein product fused to the C-terminal region of β-galactosidase, the other to give a protein product which is encoded for almost entirely by S2 DNA with the vector providing codons for initiation and termination of translation. (i) The plasmid pUR289 described by Ruther and Muller-Hull (1983) allows genes to be cloned such that the protein products are fused to the C-terminal region of β-galactosidase. The *Bam*HI fragment (935 bp) of ORF1 (Figure 1) was cloned such that it was in frame at the 3′ end of the *lacZ* gene from which 47 bases have been removed in the vector construction. The construction of this plasmid, pJM324, is described in Materials and methods. The *E. coli* strain NM522 (Gough and Murray, 1983) which overproduces the lac

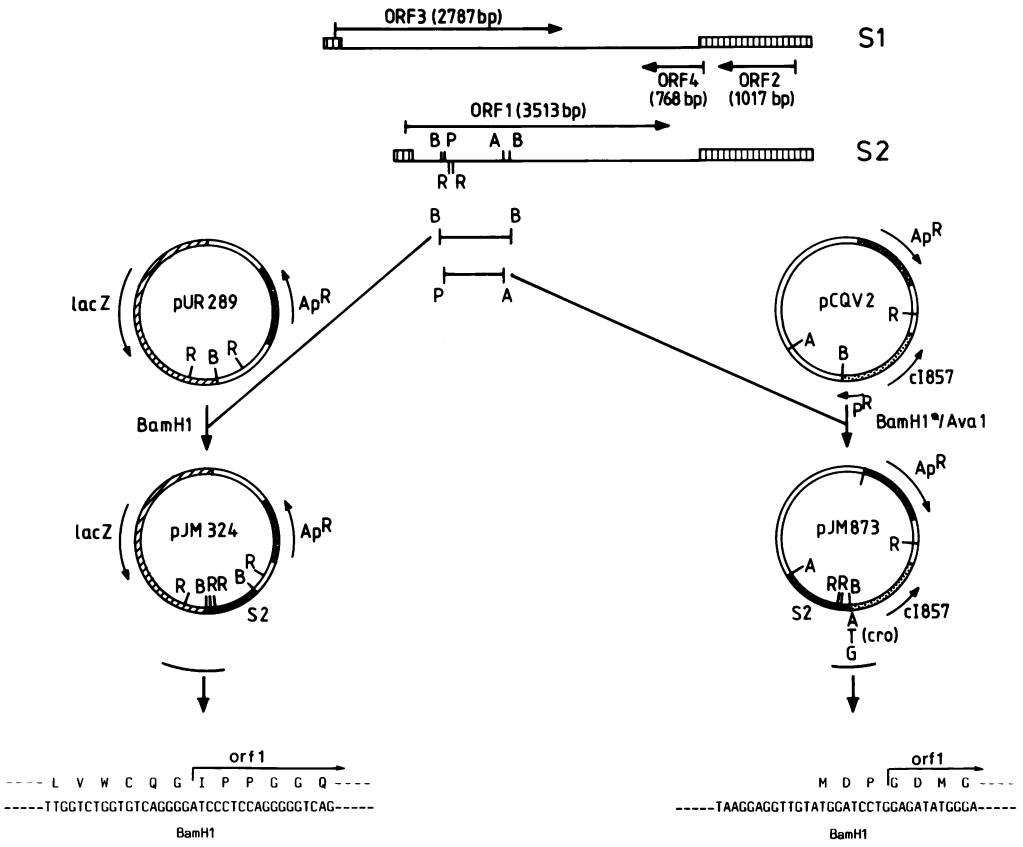

Fig. 1. Origin of recombinant plasmids directing the synthesis of ORF1 polypeptide. The upper part of the diagram shows the open reading frames of S1 and S2. ORF2 is found in the region of homology between S1 and S2 and is therefore common to both. In addition, S1 contains ORF3 and ORF4 and S2 contains ORF1. The regions of ORF1 used in the construction of the recombinant plasmids are shown. The construction of pJM324 and pJM873 from the parental plasmids pUR289 and pCQV2 are described in experimental procedures. The *Bam*HI (B) fragment of ORF1 was cloned into the *Bam*HI site of pUR289 to give pJM324. The vector pCQV2 was digested with *Bam*HI and the 3′ recessed ends filled in (*Bam*HI*). The vector was then digested with *Ava*I (A) and the *Bam*HI*/*Ava*I fragment replaced with the *Pvu*II (P)/*Ava*I (A) and the *Bam*HI*/*Ava*I fragment replaced with the *Pvu*II (P)/*Ava*I fragment of ORF1. *Eco*RI (R) fragments containing the *Bam*HI sites at the fusion point in each construct were sequenced. Part of the sequence is shown in each case to indicate the amino acids derived from ORF1. Key: ▥ regions of homology between S1 and S2; ▨ β-galactosidase gene; ▨ β-lactamase gene; ▩ lambda DNA: c1857 gene P_R promoter, ATG of *cro*; ■ S2 DNA.

repressor from I^{q1} promoter, was transformed with the plasmid. The expression can be controlled since the presence of large amounts of lac repressor will not allow expression of the plasmid *lacZ* genes in the absence of the inducer isopropyl-1-thio-β-D-galactoside (IPTG). (ii) The vector $pCQV_2$ (Queen, 1983) carries the P_R promoter of bacteriophage λ and the λ *cro* ribosome binding site. Expression is controlled by the presence of the λ c1857 gene which encodes a temperature-sensitive repressor of P_R. Genes cloned into the *Bam*HI site adjacent to the ATG of *cro* are transcribed from the P_R promoter. The *Pvu*II−*Ava*I fragment of ORF1 (788 bp) was cloned into this vector. Figure 1 shows part of the relevant S2 coding sequence present in the recombinant plasmid pJM873.

Expression of pJM324 and pJM873

To show that S2 DNA was transcribed in cells transformed with the recombinant plasmids, RNA was separated on an agarose gel, blotted on to Hybond N (Amersham International) and hybridized to a ^{32}P-labelled *Bam*HI fragment of ORF1. Cells transformed with pJM324 produced a number of transcripts ranging in size from 5 to 1.5 kb which hybridized to the probe, whereas transcripts from the vector pUR289 showed no hybridization to the S2 *Bam*HI fragment of ORF1 (Figure 2). The major transcript of 5.0 kb is sufficient to encode the expected *lacZ*/S2 protein. The minor transcripts may have arisen from incorrect initiation or termination of transcription, or as degradation products of the 5.0 kb transcript. Cells transformed with the construct pJM873 produced only one transcript that hybridized to the probe (Figure 2). This transcript was 4.5 kb in length and could therefore encode the expected S2 protein product. RNA from cells containing the vector pCQV2 showed no hybridization to the S2 *Bam*HI fragment of ORF1.

The protein products from cells carrying the recombinant plasmid pJM324 were examined in SDS polyacrylamide gels stained with Coomassie Blue. A number of proteins appear only after induction with IPTG which are not present in cells carrying the vector pUR289 (Figure 3A). These proteins were also not produced from constructs where the *Bam*HI fragment had been cloned in the opposite orientation (data not shown). The major new protein is the most abundant protein produced by the cells and has mol. wt of ~150 000 which corresponds to the size of the expected *lacZ*/S2 fusion product. The origin of the smaller polypeptides observed is unknown, but they may be degradation products of the 150 000 M_r protein or products of the truncated transcripts seen in Figure 2.

Analysis of proteins from the cells containing $pCQV_2$ and pJM873 on Coomassie-stained polyacrylamide gels revealed no differences. The proteins encoded by these plasmids were therefore analysed by two different methods. DNA from pJM873 and $pCQV_2$ were used as templates for an *in vitro* transcrip-

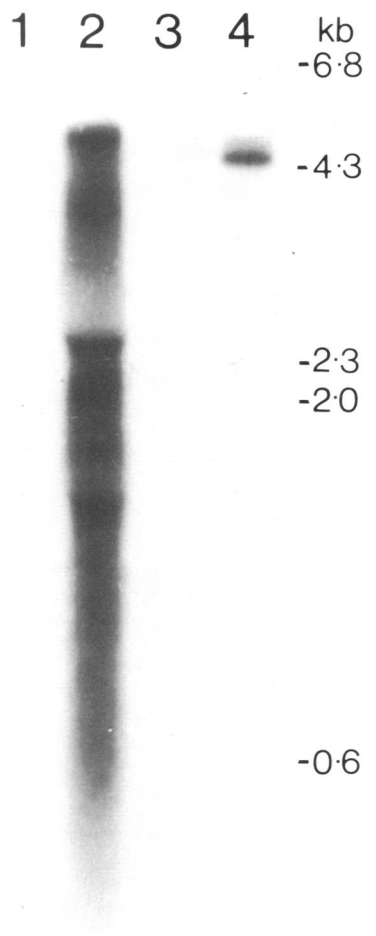

Fig. 2. Identification of ORF1-related transcripts in *E. coli* cells transformed with the recombinant plasmids. RNA was prepared from *E. coli* cells by the method of Shaw and Guest (1982) and separated on a 1.3% (w/v) agarose gel containing formaldehyde. The RNA was transferred to Hybond-N nylon membrane and probed with ^{32}P-labelled *Bam*HI fragment of ORF1. The transcripts which hybridized to the probe were visualized by autoradiography. Markers were λ DNA digested with *Hin*dIII. *E. coli* cells contained: (1) pUR289; (2) pJM324; (3) pCQV2; (4) pJM873.

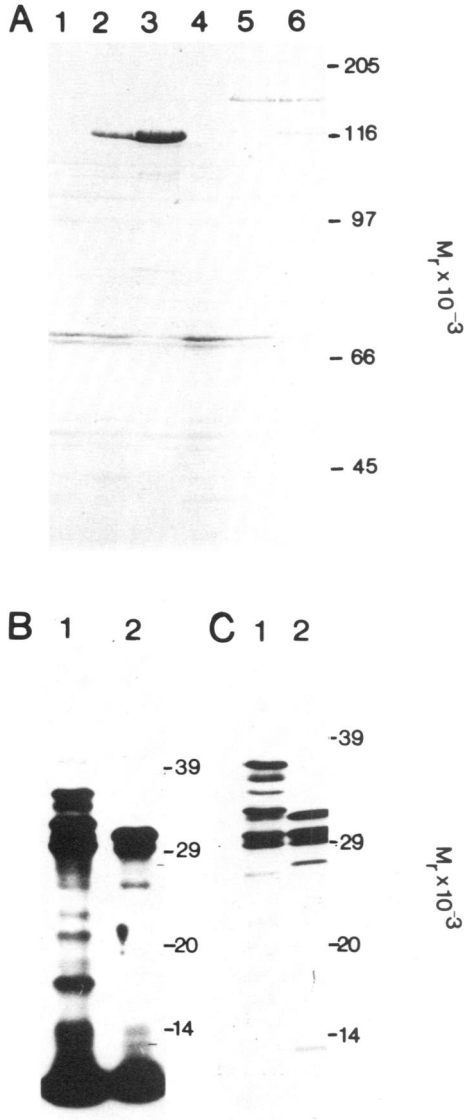

Fig. 3. Identification of polypeptides encoded by recombinant plasmids. (**A**) *E. coli* cells NM522 were transformed with pUR289 and pJM324 and grown both with and without IPTG (0.5 mM). The proteins were separated on a 10% (w/v) polyacrylamide gel containing SDS and were stained with Coomassie Blue. (**1**) pUR289 without IPTG; (**2**) pUR289 with IPTG for 1 h; (**3**) pUR289 with IPTG for 16 h; (**4**) pJM324 without IPTG; (**5**) pJM324 with IPTG for 1 h; (**6**) pJM324 with IPTG for 16 h. (**B**) Proteins encoded by pJM873 detected by transcription and translation of plasmid DNA in a cell-free system from *E. coli*. The polypeptides were labelled with [^{35}S]L-methionine and separated on a 16% (w/v) polyacrylamide gel containing SDS and visualized by autoradiography (**1**) pJM873 (**2**) pCQV$_2$. (**C**) Proteins encoded by pJM873 were also detected in minicells of WL542 transformed with recombinant plasmid. The polypeptides were labelled with [^{35}S]L-methionine and separated on a 16% (w/v) polyacrylamide gel containing SDS and visualized by autoradiography (**1**) pJM873 (**2**) pCQV$_2$.

tion–translation system derived from *E. coli* (Miller, 1972). The polypeptides synthesized were labelled with [^{35}S]L-methionine and analysed by gel electrophoresis and autoradiography. The recombinant plasmid directs the synthesis of a number of polypeptides not found in preparations using DNA from the vector pCQV$_2$ (Figure 3B). The molecular weight of the anticipated protein in this construct is 32 500 which is in good agreement with the major new protein, 32 000 M_r. The relationship between this protein and the other new proteins is unknown.

The minicell producing strain WL542 was also transformed with the plasmids pJM873 and pCQV$_2$. Plasmid coded proteins were labelled by incubation of the minicells in a medium containing [^{35}S]L-methionine and analysed by electrophoresis in SDS polyacrylamide gels followed by autoradiography. Figure 3C shows that in this system pJM873 directs the synthesis of four polypeptides not found in minicell preparations from cells transformed with the vector pCQV$_2$. The molecular weight of these polypeptides is 32 000–36 000. The relationship between these four polypeptides is also unknown but it is unlikely that the smaller ones are degradation products, since the relative amounts of each remain constant during a chase with unlabelled methionine (data not shown). The polypeptides were shown to be antigenically related (see later) and could, therefore, perhaps arise from incorrect initiation or termination of translation.

Preparation and analysis of the antiserum

The 150 000 M_r protein produced by cells containing pJM324

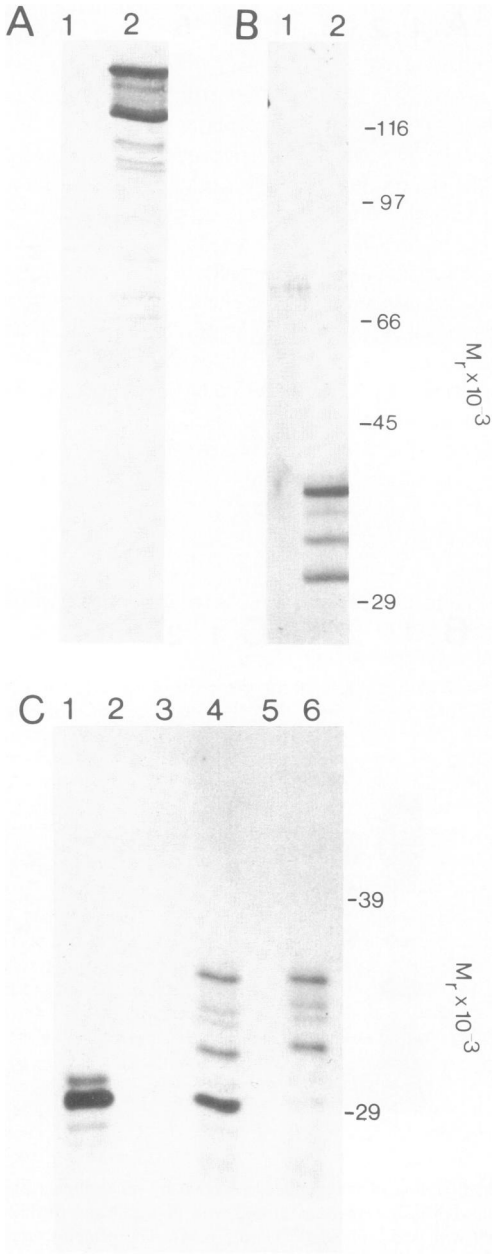

Fig. 4. Reaction of monospecific antiserum with polypeptides encoded by recombinant plasmids. (**A**) Western blot analysis of *E. coli* cells NM522 transformed with recombinant plasmids. Samples were run on a 10% (w/v) polyacrylamide gel containing SDS. After transfer to nitrocellulose the samples were probed with antiserum to the *lacZ*/S2 fusion protein which had been made monospecific to S2-derived protein by immunoprecipitation of antibodies recognizing proteins in the minicell lysates containing pUR289. The reactions were visualized by the addition of ^{35}S-labelled donkey anti-rabbit IgG followed by autoradiography. (1) pUR289; (2) pJM324. (**B**) Western blot analysis of WL542 minicells transformed with recombinant plasmids, as for (**A**): (1) $pCQV_2$; (2) pJM873. (**C**) Immunoprecipitation of proteins from minicells transformed with recombinant plasmids. Minicell proteins were labelled with [^{35}S]L-methionine and immunoprecipitated by the addition of monospecific antiserum and protein A sepharose. Samples were separated on a 16% (w/v) polyacrylamide gel containing SDS and visualized by autoradiography: (**1**) $pCQV_2$ products before treatment with antiserum; (**2**) $pCQV_2$ products precipitated with pre-immune rabbit serum; (**3**) $pCQV_2$ products precipitated with monospecific S2 antiserum; (**4**) pJM873 before treatment with antiserum; (**5**) pJM873 products precipitated with pre-immune rabbit serum; (**6**) pJM873 products precipitated with monospecific S2 antiserum.

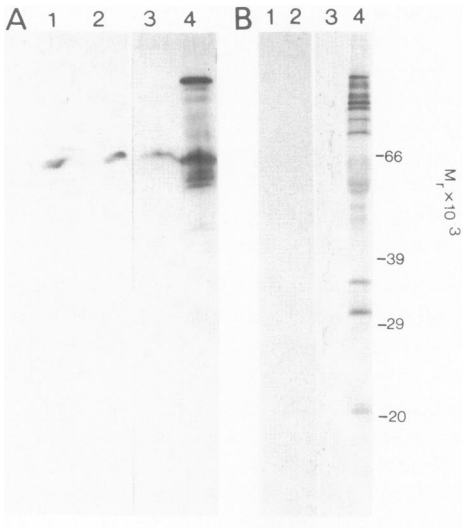

Fig. 5. Identification of mitochondrial proteins which react with monospecific antiserum. (**A**) Western blot analysis of mitochondrial proteins. Samples were run on 16% (w/v) polyacrylamide gels containing SDS. After transfer to nitrocellulose the samples were probed with monospecific antiserum and the reactions visualized by the addition of ^{125}I-labelled protein A followed by autoradiography. (**1**) B73N probed with pre-immune rabbit serum. (**2**) B73S probed with pre-immune rabbit serum. (**3**) B73N probed with monospecific antiserum (**4**) B73S probed with monospecific antiserum. (**B**) Immunoprecipitation of proteins synthesized by mitochondria. *In vitro* translation products from mitochondria labelled with [^{35}S]L-methionine were immunoprecipitated by the addition of antiserum and protein A-sepharose. Samples were run on a 16% (w/v) polyacrylamide gel and visualized by autoradiography: (**1**) B73N precipitated with pre-immune rabbit serum; (**2**) B73S precipitated with pre-immune rabbit serum; (**3**) B73N precipitated with monospecific antiserum; (**4**) B73S precipitated with monospecific antiserum.

was used for the preparation of antiserum for two reasons: it is present at much higher levels than the S2 proteins of pJM873 and, since it is a high molecular weight protein, it migrates to a region in the gel where there is less likelihood of contamination with other *E. coli* proteins.

Antibodies were made against the 150 000 M_r protein as described in experimental procedures. This antiserum reacts with numerous epitopes on the fusion protein (data not shown). In order to produce antiserum which is monospecific for epitopes of the protein encoded by S2 DNA, the serum was incubated with extracts of *E. coli* containing pUR289 and the immunoprecipitates were removed by centrifugation. This process of absorption was repeated three times more resulting in antiserum which showed no reaction against proteins from cells containing pUR289 (Figure 4A).

The antiserum was shown by Western blotting to recognize a number of polypeptides in *E. coli* lysates containing the recombinant plasmid pJM324 (Figure 4A). These proteins range in mol. wt from 150 000 (which corresponds to the size of the *lacZ*/S2 fusion protein shown in Figure 3A) to low molecular weight proteins which may represent degradation products of the fusion protein or products of the truncated transcripts shown in Figure 2. Since there is no reaction to *E. coli* lysates containing the vector pUR289, the antiserum must be reacting to the S2 epitopes of the fusion protein.

To confirm that the antiserum reacted specifically with S2-encoded protein, *E. coli* minicell lysates containing the recombinant plasmid pJM873 were separated on SDS polyacrylamide gels and blotted on to nitrocellulose. When these polypeptides

were tested against the antiserum it was shown to react with four polypeptides from minicell lysates containing pJM873 but showed no reaction to polypeptides from minicell lysates containing the vector $pCVQ_2$ (Figure 4B). These polypeptides were also immunoprecipitated by the antiserum from [^{35}S]L-methionine-labelled minicells containing pJM873 (Figure 4C). The polypeptides from minicell lysates recognized by the antiserum correspond to the four polypeptides previously identified in minicell lysates containing pJM873 (Figure 3B).

Analysis of mitochondrial protein with S2 antiserum

Having established that the antiserum was specific for S2-derived protein products it was then used as a reagent to investigate the proteins in maize. Mitochondria were prepared from 5-day-old coleoptiles of maize lines B73N and B73S and lysates were fractionated on an SDS polyacrylamide gel and blotted onto nitrocellulose. The antiserum at 1 in 150 dilution was tested against the proteins bound to nitrocellulose and the reaction visualized by addition of ^{125}I-labelled protein A. The antiserum was shown by this procedure to bind a number of proteins present in B73S but absent from B73N (Figure 5A). The major of these proteins has a mol. wt of 125 000. The antiserum bound only one protein from B73N and this reaction was shown to be non-specific since non-immune rabbit serum also bound to this protein (Figure 5A).

Mitochondrial proteins synthesized *in vitro* were labelled with [^{35}S]L-methionine and tested for reaction with the monospecific antiserum by immunoprecipitation. The products precipitated were separated on an SDS polyacrylamide gel and visualized by autoradiography. A number of labelled proteins from B73S were precipitated by the antiserum, the largest of which has a mol. wt of 125 000. No reaction was shown to B73N proteins (Figure 5B).

Discussion

The antiserum specific for the ORF1-encoded polypeptide reacted with several proteins found in B73S mitochondria which were absent from B73N. These polypeptides are of similar mol. wts to those shown to be characteristically synthesized by the mitochondria from S-type cytoplasms, the largest of which has a mol. wt of 125 000 (Forde and Leaver, 1979; Liddell and Leaver, unpublished observations). It would therefore appear that several of the differences found between *in vitro* translation products of N and S mitochondria could be accounted for in terms of translation products of ORF1. The numerous proteins reacting to the antiserum could arise from post-translational cleavage of the 125 000 M_r protein or they could represent artefacts of the *in vitro* system produced by premature termination of translation. Evidence that these proteins are not artefacts of the *in vitro* system was obtained by immunoblotting analysis of the mitochondrial proteins synthesized *in vivo* in B73S. This analysis showed a similar spectrum of cross-reacting proteins comprising a major component with M_r of 125 000 and a number of smaller polypeptides. No cross-reacting proteins were found in the B73N line.

We have shown, therefore, that the ORF1 of S2 DNA encodes a protein of 125 000 M_r and that translation products derived from ORF1 can account for a number of the differences that have been observed between S and N mitochondria of maize. The function of this protein is not yet known, but it may undergo cleavage to a number of smaller functional proteins at least some of which may be required for replication of S2, and perhaps also of S1. In this context one may recall the suggestion that S1 and S2 are of viral origin (Kemble and Thompson, 1982). Transcriptional analysis indicates one major transcript of ORF1 in mitochondria of S-type maize (Schardl *et al.*, 1985), in which case the presence of a number of mitochondrial proteins that react with the antiserum (in both *in vivo* and *in vitro* analyses) suggests that the 125 000 M_r protein may undergo post-translational modification. This resembles the situation in a number of viruses where translation of the viral RNA from a single initiation site is followed by cleavage of the large polypeptide to generate the virion proteins; several plant viruses, such as cow pea mosaic virus and animal picornaviruses such as poliovirus process functional polypeptides from a protein precursor. S1 and S2 resemble these viruses in having a protein attached to the 5′ end of the genome.

Now that antiserum specific to the ORF1 products is available it will be possible to explore the distribution and role of these mitochondrial proteins.

Materials and methods

Bacterial strains

Bacterial strains used in this work were WL542 (minA minB) and NM522 (hsd F′) (Gough and Murray, 1983).

Cloning and sequencing of DNA fragments

Mitochondrial DNA was isolated from coleoptiles of maize line B73S after 5 days germination (Forde *et al.*, 1978). S2 DNA was fractionated by electrophoresis on a 0.7% (w/v) agarose gel and purified by electroelution (Maniatis *et al.*, 1982). Fragments were cloned into M13 bacteriophage vectors mp8 and mp9 (Messing and Vieira, 1982) and mp18 and mp19 (Messing, 1983). Single-stranded templates were prepared and sequenced by the dideoxy chain termination procedure (Sanger *et al.*, 1977) using [α-^{35}S]dATP (Amersham International, 400 Ci/mmol) and buffer gradient gels (Biggen *et al.*, 1983).

Construction of plasmid pJM324

ORF1 of S2 contains a *Bam*HI fragment of 935 bp (see Figure 1) which codes for 311 amino acid residues. The *Bam*HI fragment, which had previously been cloned into M13 mp8 for sequence analysis, was removed by digestion with *Bst*I, fractionated by electrophoresis on a 1% (w/v) agarose gel, and purified by electroelution (Maniatis *et al.*, 1982). The fragment was inserted into the *Bam*HI site of pUR289 (Ruther and Muller-Hill, 1983) at the 3′ end of the *lacZ* gene. The *E. coli* strain NM522 was transformed with the plasmid and colonies were screened by hybridization (Grunstein and Hogness, 1975) using a ^{32}P-labelled S2 *Bam*HI fragment as a probe. The orientation of the insert was determined by *Eco*RI digestion of the recombinant plasmid (pJM324) and the sequence of the construct was confirmed by cloning the *Eco*RI fragment covering the *lacZ*/S2 fusion (see Figure 1) into m13 mp18 and sequenced as cited above.

Construction of plasmid pJM873

The *Pvu*II−*Ava*I fragment (788 bp) of ORF1 was purified from an agarose gel as described above. The expression vector $pCQV_2$ (Queen, 1983) was digested with *Bam*HI and the 3′ recessed ends were filled in using the Klenow fragment of *E. coli* DNA polymerase I (Maniatis *et al.*, 1982). The vector was then digested with *Ava*I. The *Pvu*II−*Ava*I fragment of S2 was ligated into the vector and *E. coli* strain WL542 was transformed with the recombinant plasmid, pJM873. The sequence of the construct was confirmed by cloning the *Eco*RI fragment covering the *cro*−S2 fusion into M13 mp18 and obtaining nucleotide sequence as described above.

RNA preparation and analysis

RNA was prepared from *E. coli* by extraction with hot phenol (Shaw and Guest, 1982) and from mitochondria as described by Koller *et al.* (1982). RNA (10 μg) was separated on a 1.3% (w/v) agarose gel containing 6.5% (v/v) formaldehyde (Maniatis *et al.*, 1982). The gel was washed for 1 h in 1 M ammonium acetate, pH 8. The RNA was transferred to Hybond-N nylon membrane (Amersham International) by the dry blot method of Smith and Summers (1980) and the filter was irradiated with u.v. light for 5 min. The filter was prehybridized for 6 h at 37°C in a buffer containing 4 × SSCP (0.48 M NaCl, 10 mM trisodium citrate, 80 mM sodium dihydrogen orthophosphate), 1 × Denhart's reagent, 50% formamide and 50 μg/ml sonicated denatured calf thymus DNA. Hybridization was carried out in the same buffer. The ^{32}P-labelled probes were denatured at 100°C for 10 min, cooled on ice and added to the hybridization buffer. Filters were hybridized at 37°C for 20 h. The filters were washed twice in 2 × SSCP/0.1% (w/v) SDS for 15 min at 37°C and twice in 0.1 × SSCP/0.5% (w/v) SDS for

30 min at 37°C. The filters were exposed to pre-flashed X-ray film at −70°C using an intensifying screen.

Preparation of hybridization probes

Hybridization probes were prepared from restriction endonuclease fragments separated in low melting point agarose gels and labelled with [α-^{32}P]dCTP, using random oligonucleotides as primers and the Klenow fragment of *E. coli* DNA polymerase I as described by Feinberg and Vogelstein (1983).

Analysis of proteins encoded by pJM324

NM522 transformed with pJM324 were grown at 37°C in L-broth medium [1% (w/v) Bacto tryptone, 0.5% (w/v) yeast extract; 0.5% (w/v) NaCl] containing 100 μg/ml ampicillin, IPTG was added to a concentration of 0.5 mM. Cells were harvested after 1−20 h induction, resuspended in 1% (w/v) SDS, 10% (v/v) glycerol 62.5 mM Tris−HCl, pH 6.8, 60 mM dithiothreitol, boiled for 5 min and electrophoresed on an SDS-polyacrylamide gel (10% w/v) according to Laemmli (1970).

Analysis of proteins encoded by pJM873

Preparation of E. coli minicells and labelling of plasmid coded proteins in vivo. The minicell strain WL542 was used as a host for the plasmids. Minicells were prepared as described by Johnson *et al.* (1981) and resuspended in M9 salts containing 30% (v/v) glycerol to a concentration of 2 × 10^{10} minicells/ml. The minicells were labelled by pelleting (200 μl) and resuspending in an equal volume of M9 salts [containing 0.2% (w/v) glucose, 0.2 g/ml vitamin B_1 and 1 mM $MgSO_4$]. After incubation at 28°C for 1 h, 20 μl of methionine assay medium (Difco) containing 20 μCi of [^{35}S]methionine (1000 Ci/mmol; The Radiochemical Centre, Amersham) were added and the reaction mixture incubated at 42°C for 15 min and 37°C for a further 1 h. The minicells were harvested by centrifugation and analysed by SDS−polyacrylamide gel electrophoresis as described above.

Labelling of plasmid coded proteins in vitro. Recombinant plasmid DNA (10 μg) was used as a template in a cell-free system involving simultaneous transcription and translation. The cell extract was prepared from *E. coli* CSH73 according to Miller (1972). The polypeptides produced were detected by labelling with [^{35}S]L-methionine and analysed electrophoretically as described above.

Preparation of antiserum

The *lacZ*/S2 polypeptide was recovered from *E. coli* containing pJM324 by preparative electrophoresis on SDS−polyacrylamide gels (10%). The *lacZ*/S2 fusion protein was identified in stained gels and this portion of unstained gels was removed and homogenized in an equal volume of phosphate-buffered saline. The homogenate (100 μg protein) was emulsified with Freund's complete adjuvant (1:1) and injected into New Zealand white rabbits. After six weeks the rabbits were injected with an emulsion of the homogenate and Freund's incomplete adjuvant (1:1). The animals were bled 10 days after the second injection. The antiserum was absorbed with proteins from *E. coli* containing pUR289 as follows: *E. coli* containing pUR289 were lysed by the addition of SDS to 1% and boiling for 5 min. The cells were diluted to 0.1% SDS and added to an equal volume of antiserum. This was incubated at 4°C for 16 h and the immunoprecipitates removed by centrifugation. This procedure was repeated until the antiserum showed no reaction to pUR289 on immunoblotting (see below).

Analysis of antiserum

Immunoblotting. E. coli proteins were transferred from SDS−polyacrylamide gels to nitrocellulose electrophoretically as described by Towbin *et al.* (1970). Non-specific binding sites were blocked by incubating the filter at 37°C for 8 h in 0.15 M NaCl, 10 mM Tris−HCl, pH 7.4 (TS) containing 1% (w/v) gelatin. The filter was then incubated overnight at room temperature to antiserum diluted 1 in 500 with 1 × TS, 0.25% (w/v) gelatin followed by 5 washes in 1 × TS, 0.1% (v/v) tween 20. The filter was then incubated for 90 min at RT in 1 × TS, 0.25% w/v gelatin, 0.5 μCi ^{35}S-labelled donkey anti-rabbit IgG (Amersham International, 375 Ci/mmol) followed by extensive washing in 1 × TS, 0.1% (v/v) tween 20. The filter was dried and exposed to X-ray film.

Mitochondrial proteins were transferred as above but were immunoblotted using 3% non-fat dried milk powder as a blocking agent and antiserum at a dilution of 1 in 150. The reactions were visualized using ^{125}I-labelled protein A and autoradiography.

Immunoprecipitation. Immunoprecipitations from [^{35}S]methionine-labelled *E. coli* lysates were carried out as described by Mellado and Murray (1983). Mitochondrial translation products were labelled with [^{35}S]L-methionine as described by Forde *et al.* (1978) and immunoprecipitation carried out as described by Dixon and Leaver (1982).

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